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Abstract

Parasitic nematode infections affect millions of people worldwide and are a significant cause of human and veterinary disease. Chronic infections cause debilitating health problems in humans, domestic animals, and livestock. Infections are treated using anthelmintic drugs, some target nicotinic acetylcholine receptors located in several different tissues. The exact mode of action of antinematode drugs is unknown. Research leading to better understand the mode of action is desirable to appreciate how resistance mechanisms develop. There is an urgent need for novel therapeutic agents to overcome resistance.

This study considered Anycylstoma caninum ACR-16 as a drug target and was investigated using two-electrode voltage-clamp electrophysiology. This technique allowed us to explore several agonist and antagonists of ACR-16 and their pharmacology expressed in X. *laevis* oocytes. The sequence of *Acn*-ACR-16 was compared with *Asu*-ACR-16, another homomeric nicotinic acetylcholine receptor, but widely distributed in *Ascaris* tissue. Also, the concentration-current-response relationships and the potencies of agonists are demonstrated for *Acn*-ACR-16. We concluded that *Acn*-ACR-16 was not sensitive to many of the currently used cholinomimetic anthelmintics. Though, the *A. caninum* channel was most sensitive to 3-bromocytisine unlike nicotine which was the most potent agonist for *A. suum* ACR-16 receptor. When considering antagonist pharmacology, the *A. caninum* receptor was moderately inhibited by α-BTX while Asu-ACR-16 was almost insensitive.

Keywords

nAChR, hookworms, *Acn*-ACR-16, anthelmintic, *Xenopus* expression

Abbreviations

α-BTX, α-bungarotoxin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester); BLASTP, protein–protein BLAST; DHβE, dihydro-β-erythroidine; DMPP, dimethyl-4-phenyllpiperazinium iodide; dTC, d-tubocurarine; nAChR, nicotinic acetylcholine receptor; ACh acetylcholine, 3-BC, 3- bromocytisine; *Acn*, Ancylostoma caninum; *Xle*, *Xenopus laevis*; cRNA, complementary RNA; STH, soil transmitted helminths; SEM, standard error mean; WHO, World Health Organization

Introduction

Parasitic nematodes can be gut or tissue dwelling, also known as soil transmitted helminths and filarial worms respectively. Infections caused by hookworms (mainly *Necator americanus* and *Ancylostoma duodenale*) are one of the leading neglected tropical disease, affecting approximately 500 million people worldwide; especially in the developing regions of Asia, Africa, Latin America and the Caribbean (Pullan et al. 2014; Loukas et al. 2016; P. Hotez 2008). These infections account for >4 million disability adjusted life years (DALYs) lost annually and an estimated global economic loss of over US$100 billion (Loukas et al. 2016; Bartsch et al. 2016). The blood feeding nematodes do not directly account for substantial mortality; instead the major clinical manifestation of hookworm infection are the consequences of chronic intestinal blood loss. Severe infection can result in poor iron deficiency, anemia, weight loss, abdominal pain, protein loss, and diarrhea (P. J. Hotez and Pritchard 1995; Bethony et al. 2006). Hookworm infections pose a major health threat to adolescent girls, women of reproductive age and children (Menzies et al. 2014; Brooker et al. 2008). Heavy worm burden can result in impaired physical and cognitive development in children and poor outcomes for pregnant women and their newborns (Guyatt et al. 2000; P. J. Hotez et al. 2014; de Silva et al. 2003).

Current hookworm control strategies are limited to deworming of infected people using anthelmintic drugs combined with ancillary strategies such as improvement of (Albonico et al. 2003) water, sanitation and hygiene (WASH) in endemic regions (WHO 2015; Campbell et al. 2018). At this time, there is no effective vaccine for human use in medical circulation (Hewitson and Maizels 2014; Diemert et al. 2008), with limited number of drug options. Unfortunately, failure of mass drug administration in prevention of hookworm transmission in endemic regions

due rapid reinfection rate, diminished efficacy or resistance of anthelmintic agents combined with the fact that adult hookworms can survive up to 7 years in the human gut producing thousands of ova per day complicates the issue (Albonico et al. 1995; Bennett and Guyatt 2000; Knopp et al. 2012; Albonico et al. 2003). Due to all of these contributing factors, novel drug targets and drugs are required to for efficient of parasitic infections.

Research has focused on several different parasitic ion channels because they are a major target binding site of most classes of antinematodal agents (Wolstenholme 2011). Ion channels are essential for fundamental physiological functioning in STHs. Nicotinic acetylcholine receptors (nAChRs) which belong to the cys-loop ligand gated ion channel family serve as synaptic transmission proteins and mediate fast transduction of signals by opening an intrinsic ion channel. They form pentameric channels which can be homomeric or heteromeric that compose a central pore. Nicotinic anthelmintics such as pyrantel, levamisole and oxantel selectively paralyze nematodes by activating cholinergic ion-channels (nAChRs) in their body wall musclulature (Williamson et al. 2009; Abongwa et al. 2017). The significance of nematode nAChRs as drug targets has been underlind by the recent development of novel amino-acetonitrile compounds (Kaminsky et al. 2008).

Ancylostoma caninum is the most widespread and pathogenic hookworm of dogs (Nemzek et al. 2015). Infestation typically results in anemia with bloody diarrhea, hemorrhagic enteritis, vomiting, anorexia, dehydration and poor weight gain, sometimes leading to death (Epe 2009; Dias et al. 2013). Zoonotic infection with *A. caninum* in humans has been associated with eosinophilic enteritis, localized myositis and cutaneous larva migrans (Prociv and Croese 1996; Bowman et al. 2010; Landmann and Prociv 2003; Traversa 2012). *Amcylostoma caninum* is most accessible of all hookworms and is closely related to human hookworm species, *Ancylostoma duodenale* and *Necator americanus*. Therefore, they are used as a model for human hookworm.(Nemzek et al. 2015; Prociv and Croese 1996; Blaxter 2000).

In this study, a homologue ACR-16 was cloned and expressed from *Ancylostoma caninum*, clade V nematode parasite. The receptors were expressed in *Xenopus laevis* oocytes and we used two-electrode voltage-clamp to understand the pharmacology. ACR-16 has been validated as a target in parasitic clade III roundworm, *Ascaris suum* by Abongwa et al. (2016). The focus of this study

was to generate a comparative pharmacological analysis of the homomeric channel and establish ACR-16 as a valid target in the hookworm parasites.

Materials and Methods

Ethical concerns

No vertebrate animals were used directly in this study. *Ancylostoma caninum* larvae were a kind gift from Dr. Matt Brewer (Iowa State University, Ames, IA). Defolliculated *Xenopus laevis* oocytes were obtained from Ecocyte Bioscience (Austin, TX, USA).

Sequence Analysis

Database searches for *A. caninum* ACR-16 were performed with the BLAST search (WormBase Parasite), using the BLASTP algorithms (Altschul et al. 1997). Signal peptide predictions were done using the SignalP 4.1 server (Petersen et al. 2011), and membrane-spanning regions were identified using TMpred (Hofmann and Stoffel 1993). Alignment of the full-length amino acid sequences with *Ascaris suum* ACR-16 was carried out using Clustal Omega program (Sievers et al. 2011).

Cloning of Acn-ACR-16

TRIzol Reagent™ (Invitrogen™, Carlsbad, CA, USA) was used to extract total RNA from *A. caninum* larvae. cDNA was synthesized by using SuperScript VILO Master Mix ((Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions and served as a template for the amplification of multiple fragments of the *Acn*-ACR-16 sequence (WormBase Parasite Gene ID: ANCCAN_01899). We used Gibson assembly protocol to assemble the amplified fragments into the full length *Acn*-ACCR-16 sequence (Gibson et al. 2009). Full length product was subcloned into pTB207 expression vector by adding *XhoI* and *ApaI* restriction enzyme sites respectively to the forward primer (5' end: TGGCGGCCG*ctcgag*ATGCGTTCGTTGGTCGTCTG) and reverse primers (3' end: ATCAAGCTC*gggccc*TTAGGCGACGAGATATGGAGC) using In-Fusion cloning (Takara Bio USA, Inc.). The final cloned constructs were sequenced with pTB207 vector primers (forward, T7) and (reverse, SP6). Only positive clones were used for cRNA synthesis using in vitro transcription with the mMessage mMachine T7 transcription kit (Ambion) and the cRNA was aliquoted and stored at -80°C.

Oocyte Microinjection

Defolliculted *Xenopus laevis* oocytes were injected with 25-50 ng of *Acn*-ACR-*16* cRNA either alone or in combination with 15-25 ng of each ancillary (*A. suum ric*-3, *unc*-50 and *unc*-74) in a total volume of 50 nL. Incubation solution consisting of 100mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2.H_2O$, 1 mM $MgCl_2.6H_2O$, 5 mM HEPES, 2.5 mM pyruvate, 100 $U•mL^{-1}$ penicillin, and 100 $ug•mL^{-1}$ streptomycin (pH 7.5) was used to store *Xenopus* oocytes at ~19˚C for approximately 3 hours prior to injections. The oocytes were injected at the animal pole using a nanoject II microinjector (Drummond Scientific, Broomall, PA, USA) and then transferred into a 96-well culture plate. In each well, 200 µL of incubation solution was placed with one oocyte and and the solution was changed daily. The oocytes were incubated at 19°C for for 2-7 days. This time period allowed for robust receptor expression.

Two-Electrode Voltage Clamp Electrophysiology in Xenopus Oocytes

Two-electrode voltage clamp electrophysiology recordings from *Acn*-ACR-16 expressed were performed as described by Abongwa et al. (2016) . BAPTA-AM, a calcium chelator, was added ~3 hours prior to recordings to prevent endogenous calcium channel activated chloride channels. Non-injected oocytes served as negative control during recordings. The recordings were made using Axoclamp 2B amplifier (Warner Instruments, Hamdem, CT, USA) with oocyte clamped at -60 mV and Data were collected using Clampex 10.2 (Molecular devices, Sunnyvale, CA, USA). Microelectrodes were used for impaling oocytes were used via Flaming Brown horizontal electrode puller (Model P-97, Sutter Instruments Co., USA) and filled with 3 M KCl. The microelectrodes tips were carefully broken with a tissue paper to achieve a resistance of 2-5 MΩ in recording solution (100 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2·2H_2O$ and 5 mM HEPES, pH 7.3) in order to allow passage of large currents for maintaining adequate voltage clamp.

Drug applications

All drugs applied, except tribendimidine and derquantel, were purchased from Sigma Aldrich (St Louis, MO, USA). The drugs were solubilized in recording solution or DMSO (final working concentration did not exceed 0.1%). Derquantel and tribendimidine were a generous gift from Zoetis (Kalamazoo, MI, USA) and Prof Shu Hua Xiao (National Institute of Parasitic Diseases, China) respectively.

Agonists of interest were used at a final concentration of 100 µM except tribendimidine (30 µM) due to solubility issues. In all experiments, 100 µM acetylcholine was applied first and all the responses were normalized to this control response. Each agonist was applied for 10s followed by 3 minutes perfusion with recording solution. The sequence for application of agonists for determining the potency series was random and not predetermined. The concertation-response studies were conducted by application of the drug in ascending order of concentrations in order to minimize the desensitization by high concentrations of agonists. In each experiment the drug was applied for 10s followed by 3 minutes wash off with recording solution.

All the antagonists in our study were used at a final concentration of 10 µM. For generating rank order potency series for the antagonists, a control application of 100 µM ACh was first initially followed by 3 minutes wash off. Thereafter, 100 µM acetylcholine was applied for 10 s, immediately followed by 10 s application of the antagonist in the continued presence of 100 µM ACh and then a final 10 s application of 100 µM ACh. At least 3 min drug wash off interval was allowed between applications in order to minimize desensitization. Note that, due to short time of drug application in this protocol, it is possible to underestimate the potency of an antagonist.

Data and Statistical Analysis

Clampfit 10.3 (Molecular Devices, Sunnyvale, CA, USA) and GraphPad Prism 7.0 (Graphpad Software Inc., La Jolla, CA, USA) were used to analyze data. The peak currents in response to the applied agonists were measured and normalized to control current (100 µM acetylcholine). All completed drug application sequences on the oocytes were used for analysis without exclusion. If the recording became unstable, indicated by a change in the baseline holding current, all of that recording was rejected for analysis. The results expressed as mean ± SEM. The Hill equation was used to analyze the dose-response relationships by fitting log dose-response data points as described in (Boulin et al. 2008). Desensitization kinetics in response to the agonists were fitted using a single exponential decay fit:

$$f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau i + C}$$

where n is the number of components, A is the amplitude, t is time, τ is the time constant, and C is the constant *y*-offset for each i component. The mean % inhibition produced by the antagonists on currents elicited by 100 μM acetylcholine were calculated using the equation:

$$Inhibition\ (\%) = (1 - \frac{Iant}{\frac{Iant\ control}{Imax\ control} \times Imax}) \times 100\%$$

where $Imax\ control$ is the peak current of the control 30 s application of 100 μM ACh, $Imax$ is the peak current of the 100 μM ACh that preceded the 10 s co-application of ACh and antagonist. $Iant$ is the minimal current during the co-application of 100 μM ACh and the antagonist. $Iant\ control$ is the current at the same point from the beginning of the 30 s application as $Iant$ during the control 30 s application of 100 μM ACh. Statistical analyses were performed using *t*-test and one-way ANOVA. We defined $P<0.05$ as showing statistical significance.

Results

Sequence comparison of Acn-ACR-16 with Asu-ACR-16

We were able to identify the potential complete coding sequence of the homolog of ACR-16 in the dog parasite (Gene ID: ANCCAN_01899) by using the *Asu*-ACR-16 protein sequence (GeneBank: KP756901) as a query in a BLASTP search in the nematode protein database, WormBase Parasite. *Acn*-ACR-16 has all the structural characteristics of a nicotinic acetylcholine receptor subunit : a large extracellular NH_2-terminal domain of ~200 amino acids involved in correct nAChR assembly, Cys-loop motif separated by 13 intervening amino acids, four transmembrane (TM) domains that forms the ion-conducting pore, a cytoplasmic domain inserted between TM3 and TM4 that helps in modulation of channel activity & ion conductance, six loops (A-F) and most importantly presence of vicinal cysteines (Y-x-C-C motif) in the C-loop making an alpha subunit. Figure 1 shows the protein sequence alignment of *Acn*-ACR-16 with *Asu*-ACR-16. Both the worms belong to different clade of nematodes, but their amino acid residues were highly conserved with an identity of 77.8% identity. There is lack of conservation in *Acn*-ACR-16 loops E and F which encouraged us to characterize the pharmacology of the ion channel.

Acn-ACR-16 forms 3-bromocytisine sensitive nAChR

We tested a selection of nicotinic agonists and cholinergic anthelmintics on the expressed *A. caninum* ACR-16 ligand gated ion channel. Figure 2A shows the rank order potency series for the agonists along with the representative traces. 3-Bromocytisine was the most potent agonist were the most potent (>130% of the acetylcholine current). Epibatidine, cytisine, nicotine and DMPP activated the receptor. Interestingly levamisole, oxantel, pyrantel, morantel, bephenium, and tribendimidine were not active on the expressed nAChR. The rank order potency series on *Acn*-ACR-16 when normalized to the control 100 μM acetylcholine current is: 3-bromocytisine > ACh > epibatidine > cytisine > nicotine > DMPP >> levamisole = oxantel = pyrantel = morantel = choline = bephenium = tribendimidine. None of cholinomimetic anthelmintics currently used in the field activated the homomeric receptor which shows that this channel is distinct from the other somatic nAChRs of the nematodes.

Comparative pharmacology of acetylcholine and 3-bromocytisine

The concentration-response relationship was examined by application of drugs in ascending order (0.3-300 μM depending on the agonist). Figure 2B shows the representative recordings and concentration response curve induced by the application of acetylcholine and 3-BC. The initial application of 100 μM acetylcholine was used as an internal standard for normalization for each recording. The sigmoidal concentration-response fit gave an EC_{50} = 49.7 ± 3.3 μM for acetylcholine. 3-Bromocytisine produced a considerable left shift in the dose-response curve and gave a significantly lower EC_{50} = 1.5 ± 0.4 μM for 3-BC. The curves for both the nicotinic agonists were steep but the hillslope (n_H) values were not different significantly (n_H = 2.5 ± 0.3 for ACh and 2.4 ± 0.7 for 3-BC). The steep dose-response curves and the higher hill slope values (>1) suggests that the ligands are binding to more than one site in the receptor and exhibits positive cooperativity as expected of a homomeric ion channel.

Acn-ACR-16 desensitization

Desensitization defined as decrease or loss of biological response following prolonged or repetitive stimulation, is a common feature of the nAChRs including α-7 homomeric nAChRs (Giniatullin et al. 2005; Picciotto et al. 2008; Quick and Lester 2002). In case of *Ascaris suum* ACR-16, all the

potent agonists exhibited desensitization (Abongwa et al. 2016). We observed similar trend characterized by the peak and waning current responses observed during maintained (for 10 s) agonist applications in case of *Acn*-ACR-16. Bar graph in figure 2C shows the time constants for desensitization observed with agonists. The time constant for desensitization was highest for epibatidine and lowest for 3-bromocytisine. The mean time constants for desensitization rates ranged between 1.5 and 4.8 s for of *Acn*-ACR-16 and were less than the rates observed in *Asu*-ACR-16 (Abongwa et al. 2016).

Ancillary factor ric-3 is required for functional expression of Acn-ACR-16

For the heterologous expression of the *Acn*-ACR-16, we expressed the subunit protein cRNA with different ancillary proteins. Figure 3 shows the effects of co-injecting different ancillary proteins (*ric-3*, *unc-50* and *unc-74* from *A. suum* and *ric-3* from *X. laevis*) in combination with ACR-16 cRNA. None of the combinations, except *Acn*-ACR-16 with *Asu-ric*-3, gave robust responses to control 100 μM ACh. In order to optimize the expressed we varied the amount of cRNA of *Acn*-ACR-16 (25-50 ng) and *Asu-ric-3* (15-25 ng). We measure the largest response from oocytes injected with 50 ng *Acn*-ACR-16 and 25 ng *Asu-ric-3* and this mix was used for all subsequent recordings.

Antagonist pharmacology

Six nAChR antagonists (10 μM each) were tested on the expressed cation selective homomeric ion channel. The antagonists were d-tubocurarine (dTC), mecamylamine, dihydro-β-erythroidine (DHβE), derquantel , hexamethonium, and α-bungarotoxin (α-BTX). We measured the mean % inhibition of the control 100 μM ACh current to determine the rank order potency of the antagonists. αBTX produced least inhibition of the acetylcholine mediated current while d-TC and mec were most potent (~100% inhibition of the control current). DhβE, a selective antagonist for $\alpha_4\beta_2$ receptors (Levin 2002), interestingly produced almost complete inhibition of acetylcholine currents. The complete rank order potency for antagonists (Figure 4) was: dTC ≈ mecamylamine ≈ DHβE > derquantel > hexamethonium > α-BTX.

Discussion

Comparison of pharmacology of Acn-ACR-16 with Asu-ACR-16

In this study we have shown that ACR-16 from *Ancylostoma caninum*, the dog hookworm, expresses as a homomeric channel in *Xenopus* oocytes. Abongwa et al. (2016) successfully recapitulated and validated ACR-16 homolog from the pig parasite, *Ascaris suum*. Similar to *A. suum* channel, the *Acn*-ACR-16 was not sensitive to many of the currently used cholinomimetic anthelmintics including levamisole, pyrantel and tribendimidine. However, *A. caninum* homomeric cys-loop channel was most sensitive to 3-bromocytisine while nicotine was the most potent agonist for *A. suum* ACR-16 receptor. The concentration response curves for acetylcholine was slightly steeper for *Asu*-ACR-16 (n_H = 3.9 ± 0.3) in comparison to *Acn*-ACR-16 (2.5 ± 0.3); which may account for higher sensitivity of acetylcholine for the pig nematode alpha nAChR (*Asu*-ACR-16's EC_{50} = 4.5 ± 0.2 μM; *Acn*-ACR-16's EC_{50} = 49.7 ± 3.3 μM). In terms of antagonist pharmacology, the *A. caninum* cation channel was moderately inhibited by α-BTX (mean % inhibition = 49.3 ± 5. 2) while Asu-ACR-16 was nearly insensitive (mean % inhibition = 5.5 ± 0.8). In addition, DhβE produced complete inhibition of acetylcholine mediated responses on the *Acn*-ACR-16's while *A. suum* homolog was moderately inhibited. In agreement with Abongwa et al. (2016), *Acn*-ACR-16 was highly sensitive to mecamylamine & d-TC and moderately sensitive derquantel & hexamethonium. The protein sequence of the ACR-16 homologues from both the nematode parasites is highly identical (77.8 % identity). But there are variable amino acids residues in the loops E and F which can account for differences in the pharmacological properties (Corringer et al. 2000).

Consideration of Acn-ACR-16 as a drug target

Hookworms infections affects approximately 500 million people with 5.1 billion at risk for acquiring infection worldwide (Global Burden of Disease Study 2015; Pullan and Brooker 2012). Despite decades of strong research efforts and identification of promising candidate antigens, there are still no commercially available vaccines for human hookworm infections. Consequently, identification of novel drug targets is a practical and feasible approach to control these infections. In parasitic nematodes nicotinic acetylcholine receptors are required for various physiological functions. These ligand-gated ion channels are targets of important cholinergic antinematodal drugs such as levamisole & pyrantel and recently introduced "novel" anthelmintics including

tribendimidine & derquantel. ACR-16 has been shown to be an important for regulation of fast neurotransmission in *C. elegans* (Richmond and Jorgensen 1999). *Asu*-ACR-16 has been validated as a drug target in the pig parasitic nematode (Abongwa et al. 2016). We have successfully reconstituted a fully functional homomeric nAChR, ACR-16, in the *Xenopus* oocyte expression system from *A. caninum*, which is used as a model for human hookworm infections. The pharmacology of the receptor is distinct from the levamisole sensitive nematode receptors. The ACR-16 homologue also displayed some pharmacological differences from *Asu*-ACR-16. Benzimidazoles are the commonly used antiparasitic drugs for treatment of hookworm infections but there have been multiple reports of resistance in veterinary medicine and decreased cure rates in humans (Geerts and Gryseels 2000; De Clercq et al. 1997; Keiser and Utzinger 2008; Conder and Campbell 1995). There is a need for new drug target and ACR-16 may be a valid target site with the potential to circumvent resistance.

Acknowledgements

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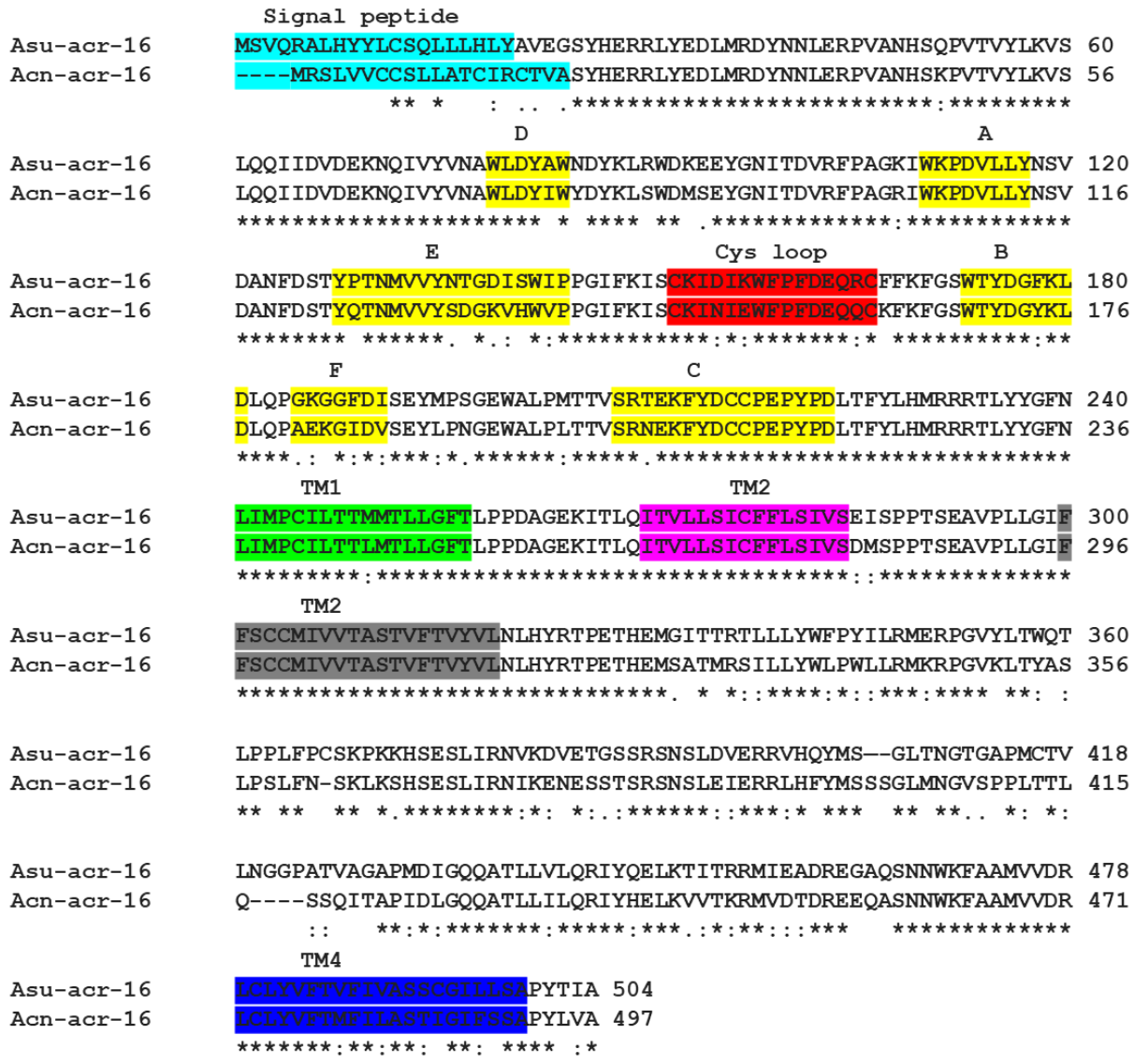


Figure 1. The amino acid sequence alignment of ACN-ACh-16 and Ascaris suum ACR-16. The signal peptide (light blue), transmembrane regions TM1-4, and cys-loop (red) are indicated. The C-binding loop consistent of adjacent cysteines in the α-subunit are indicated in the blue box.

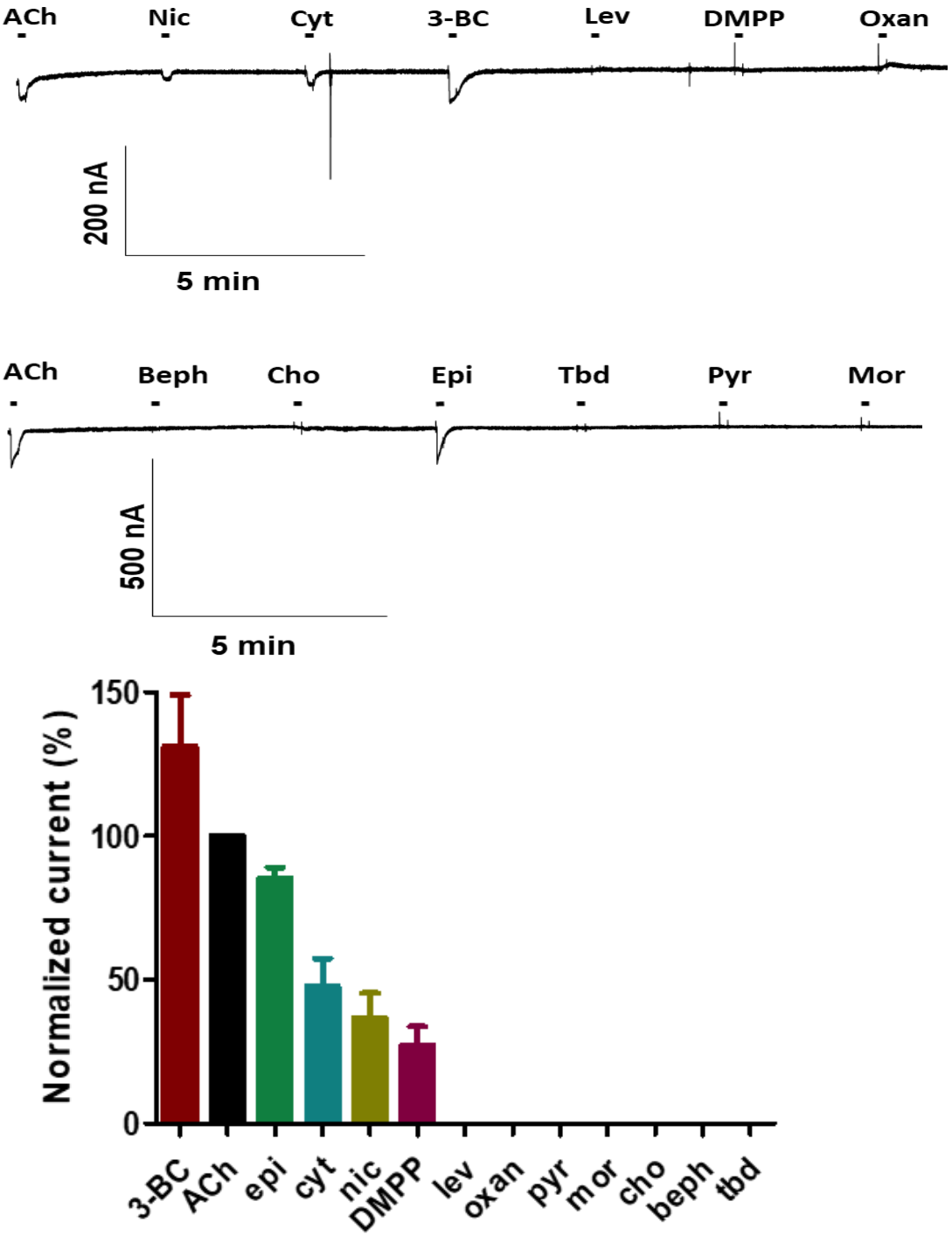


Figure 2A. The effects of nAChR agonists and antiparasitic drugs on Acn-ACR-16. Rank order potency series for nAChR agonists and anthelminitcs along with sample traces and mean ± SEM (n ≥ 4) is shown. Nicotine (nic), cytisine (cyt), 3-bromocytisine (3-bc), epibatidine (epi), DMPP, choline (cho) are measured agonists. Levamisole (lev), oxantel (oxan), pyrant (pyr), morantel (mor), choline (cho), bephenium (beph), and tribendimidine (tbd)

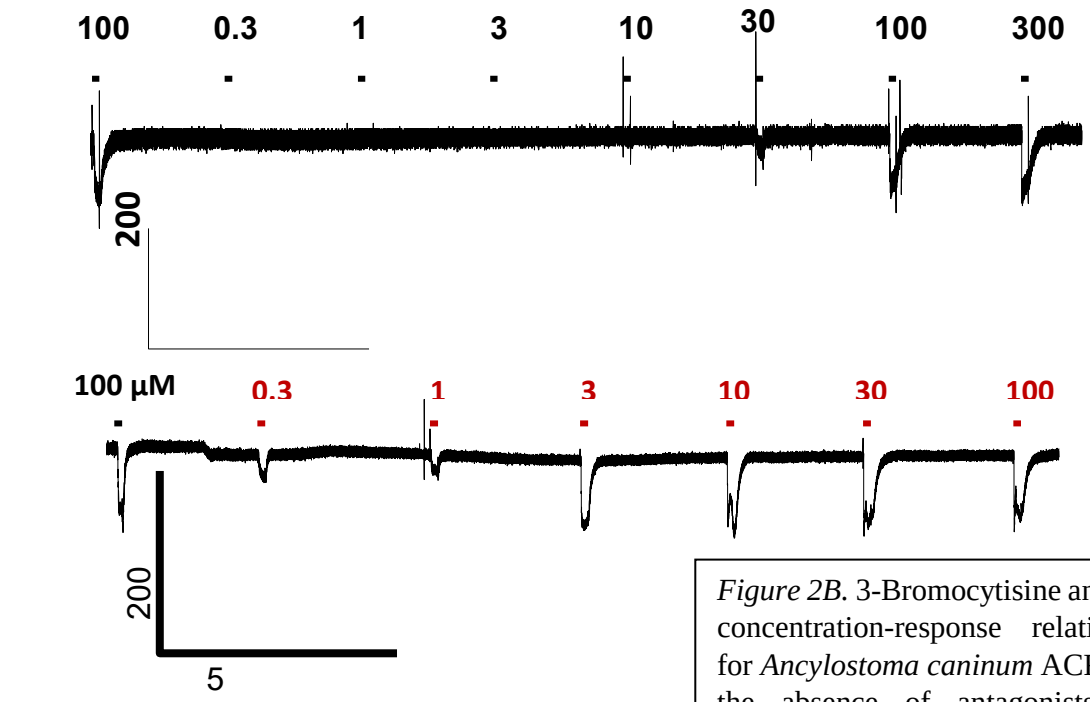
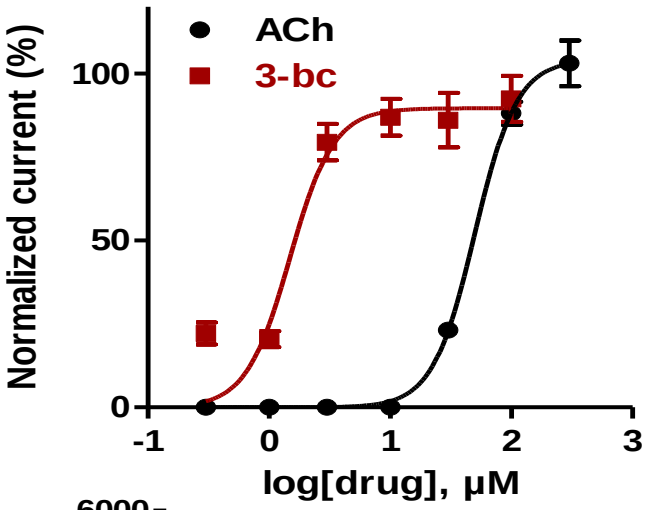


Figure 2B. 3-Bromocytisine and ACh concentration-response relationship for *Ancylostoma caninum* ACR-16 in the absence of antagonists with associated traces. ACh and 3-bc dose-response curves for Acn-ACR-16. For ACh the EC_{50} values were (mean ± SEM) 49.7 ± 3.7 and the Hill slope (n_H) = 2.5 ±. 0.3. For 3-bc the EC_{50} values were (mean ± SEM) 1.5 ± 0.35 and the Hill slope (n_H) = 2.5 ± 0.1. N=6 for both values



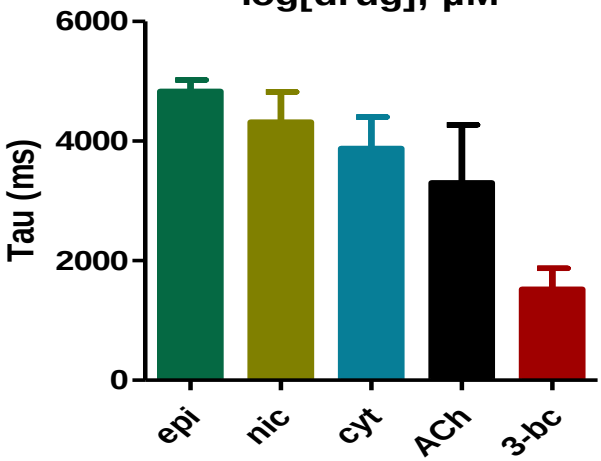


Figure 2C. Ancylostoma caninum ACR-16 desensitization rate constant fit. Desensitization responses to nicotine (nic), cytisine (cyt), 3-bromocytisine (3-bc), epibatidine (epi), and acetylcholine (Ach) of Acn-ACh-16 are represented via bar chart (mean ± SEM). The rank order for Acn-ACR-16 desensitization time constants is the following: 100 µM epi > 100 µM nic > 100 µM cyt > 100 µM ACh > 100 µM 3-bc. The desensitization rates ranged from 1150-480 ms. N= 6 for all values

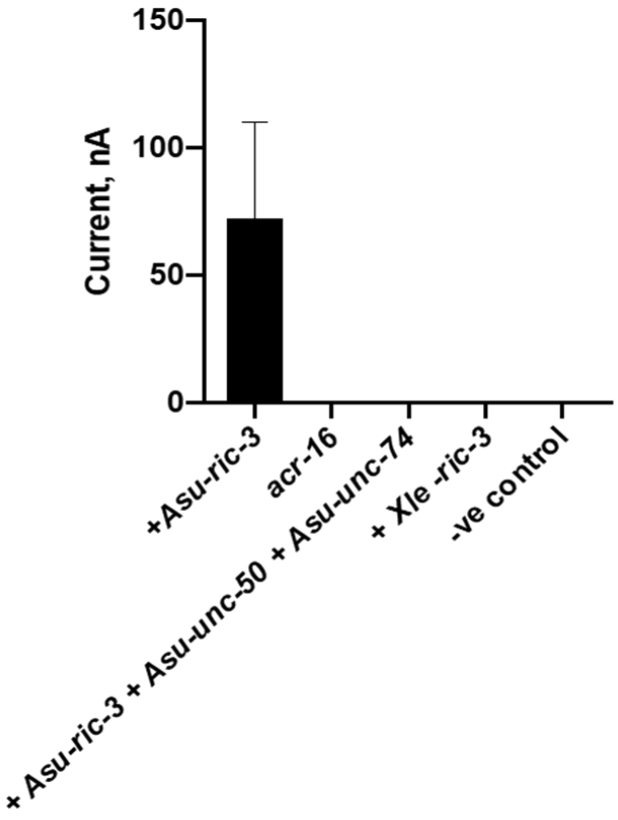


Figure 3. Effects of co-injecting different ancillary proteins (*ric-3*, *unc-50* and *unc-74* from *A. suum* and *ric-3* from *X. laevis*) in combination with ACR-16 cRNA. None of the combinations, except *Acn*-ACR-16 with *Asu-ric*-3, gave robust responses to control 100 µM ACh

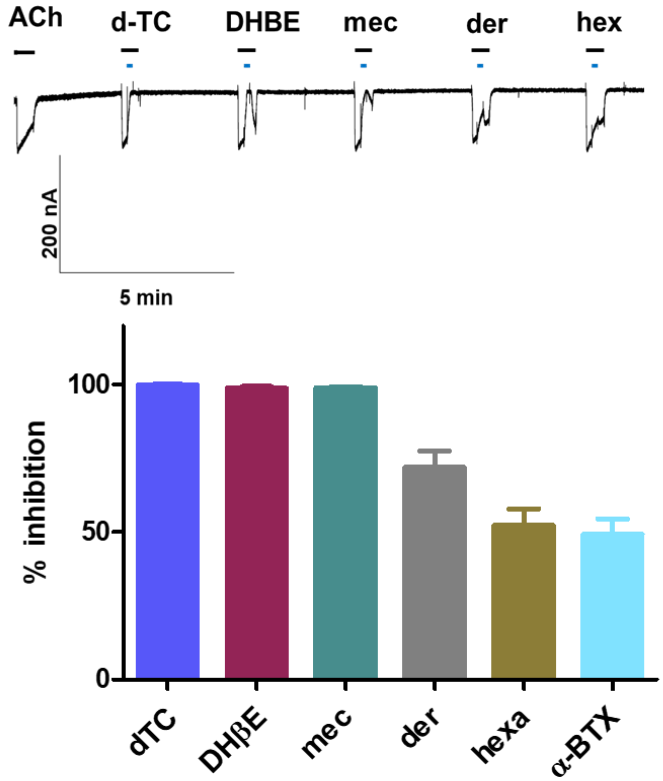


Figure 4A. Sample traces for ACh antagonists concentration-response relationships for Acn-ACh-16 in the presence of 10µM of ACh, dTC, DHBE, mec, der, and hexa.

Figure 4B. The effects of selected nAChR antagonists on *Acn*-ACR-16 mediated by ACh responses. The bar chart displays the effects of selected antagonists on ACR-16. Results are expressed as mean (± SEM) % inhibition of currents stimulated by 100 µM ACh for all antagonists. dTC, mecamylamine (mec), and DHβH completely blocked *Acn*-ACR-16 mediated responses. Derquantel (der) and hexamethonium (hexa) produced partial blocks of *Acn*-ACR-16 mediated ACh responses. While, αBTX produced the lowest block of *Acn*-ACR-16 mediated ACh responses. The rank order potency series for nAChr antagonists each tested at a concentration of 10 µM of the following: dTC ≈ DHβE ≈ mec > der > hexa > αBTX. ANOVA and Bartlett's test for variance inhomogeneity and found no significant difference and Tukey's multiple comparison tests. N=6 for all values

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